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שמסה הוץ of an invention the title of which is assignment by the inventor

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תכשירי רוקחות המכילים חומצות קרבוקסיליות ותולדותיהם

(באנגלית) (English)

PHARMACEUTICAL COMPOSITIONS CONTAINING CARBOXYLIC ACIDS AND DERIVATIVES THEREOF

מבקש בזאת כי ינתן לי עליה מסנס
hereby apply for a patent to be granted to me in respect thereof.

* בקשת פטנט מוסף — Application for Patent Addition	* בקשת חלוקה — Application of Division	* דרישה רין קדימה Priority Claim		
* לבקשה/לפטנט to Patent/Appl.	מבקשת פטנט from Application	מספר/סימן Number/Mark	תאריך Date	מדינת האגוד Convention Country
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הוגש בענין filed in case

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Signature of Applicant

COHEN ZEDEK & RAPAPORT

By: [signature]

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PHARMACEUTICAL COMPOSITIONS CONTAINING CARBOXYLIC ACIDS AND DERIVATIVES THEREOF

תכשירי רוקחות המכילים חומצות קרבוקסיליות ותולדותיהם

ABSTRACT

The orphan receptor HNF-4$_\alpha$ controls the expression of genes which govern the onset and progression of cancer, atherogenesis, hyperlipidemia, insulin resistance, hypertension, blood coagulability and fibrinolytic defects, autoimmune and other diseases. Natural and xenobiotic amphipathic carboxylates are shown here to directly modulate the transcriptional activity of HNF-4$_\alpha$ by binding of the respective acyl-CoA thioesters to the HNF-4$_\alpha$ ligand binding domain. Binding may result in either activation or inhibition of HNF-4$_\alpha$ transcriptional activity as function of chain length, degree of saturation and degree of substitution of respective acyl-CoA ligands. Inhibition of HNF-4$_\alpha$ transcriptional activity by natural or xenobiotic amphipathic carboxylates capable of being endogenously converted to their respective CoA thioesters may offer a therapeutic mode for treating diseases initiated or promoted by overexpression of HNF-4$_\alpha$-controlled genes.

Background

Hepatocyte nuclear factor-4[1] (HNF-4) (reviewed in ref. 2) is an orphan member of the superfamily of nuclear receptors. HNF-4 is expressed in the adult and embryonic liver, kidney, intestine and adipose tissue and disruption of the murine HNF-4 by homologous recombination results in embryo death. Like other members of the superfamily, the HNF-4 receptor consists of a modular structure comprising a well conserved N-terminal DNA binding domain linked through a hinge region to a hydrophobic C-terminal ligand binding domain. Two HNF-4 isoforms have been cloned and characterized: HNF-41 and HNF-42 comprising of a splice variant having a 10 amino acids insert in the C-terminal domain.

HNF-4 is an activator of gene expression. Transcriptional activation by HNF-4 is mediated by its binding as a homodimer to responsive DR-1 promoter sequences of target genes resulting in activation of the transcriptional initiation complex. Genes activated by HNF-4 (reviewed in ref. 2) encode various enzymes and proteins involved in lipoproteins, cholesterol and triglycerides metabolism (apolipoproteins AI, AII, AIV, B, CIII, microsomal triglyceride transfer protein, cholesterol 7 hydroxylase), lipid metabolism (mitochondrial medium chain fatty acyl-CoA dehydrogenase, peroxisomal fatty acyl-CoA oxidase, cytochrome P-450 isozymes involved in fatty acyl -oxidation and steroid hydroxylation, fatty acid binding protein, cellular retinol binding protein II, transthyretin), glucose metabolism (phosphoenolpyruvate carboxykinase, pyruvate kinase, HNF-1), amino acid metabolism (tyrosine amino transferase, ornitine transcarbamylase), blood coagulation (factors VII, IX, X), iron metabolism (transferrin, erythropoietin) and macrophage activation (hepatocyte growth factor-like protein/macrophage stimulating protein, Hepatitis B core and X proteins, long terminal repeat of human HIV-1, 1- antitrypsin).

Some genes activated by HNF-4$_\alpha$ play a dominant role in the onset and progression of atherogenesis, cancer, autoimmune and some other diseases[3]. Thus, overexpression of apolipoproteins B, AIV and CIII as well as of microsomal triglyceride transfer protein may result in dyslipoproteinemia (combined hypertriglyceridemia and hypercholesterolemia) due to increased production of very low density lipoproteins (VLDL) and chylomicrons combined with decrease in their plasma clearance. Similarly, HNF-4$_\alpha$/HNF-1-induced overexpression of pancreatic insulin combined with enhanced pancreatic glycolytic rates due to overexpression of pancreatic pyruvate kinase may result in hyperinsulinemia leading to insulin resistance. Indeed, mutations in HNF-4$_\alpha$ and HNF-1 were recently shown to account for maturity onset diabetes of the young (MODY)[4]. Insulin resistance combined with HNF-4$_\alpha$-induced overexpression of liver phosphoenolpyruvate carboxykinase and increased hepatic glucose production may result in impaired glucose tolerance (IGT) leading eventually to noninsulin dependent diabetes mellitus (NIDDM). Furthermore, hyperinsulinemia is realized today as major etiological factor in the onset and progression of essential hypertension and overexpression of HNF-4$_\alpha$ controlled genes may therefore further lead to hypertension. Furthermore, HNF-4$_\alpha$-induced overexpression of blood coagulation factors combined perhaps with overexpression of inhibitors of blood fibrinolysis (*e.g.,* plasminogen activator inhibitor -1) may lead to increased thrombus formation and decreased fibrinolysis with a concomitant aggravation of atherosclerotic prone processes.

Dyslipoproteinemia, obesity, IGT/NIDDM, hypertension and coagulation/fibrinolysis defects have been recently realized to be linked by a unifying Syndrome (Syndrome-X, Metabolic Syndrome, Syndrome of insulin resistance)[5]. High transcriptional activity of hNF-4$_\alpha$ resulting in overexpression of HNF-4$_\alpha$-controlled genes may indeed account for the etiological linkage of Syndrome-X categories. Syndrome-X categories and the

Syndrome in toto are realized today as the major risk factors for atherosclerotic cardiovascular disease in Western societies, thus implicating HNF-4$_\alpha$ in initiating and promoting atherogenesis. Furthermore, since breast, colon and prostate cancers are initiated and promoted in Syndrome-X inflicted individuals, overexpression of HNF-4$_\alpha$ controlled genes could be implicated in the onset and progression of these malignancies.

In addition to the role played by HNF-4$_\alpha$ in the expression of Syndrome-X related genes, HNF-4$_\alpha$ activates the expression of genes which encode for proteins involved in modulating the course of autoimmune reactions. Thus, HNF-4$_\alpha$-induced overexpression of the macrophage stimulating protein may result in sensitization of macrophages to self antigens or crossreacting antigens, thus initiating and exacerbating the course of autoimmune diseases, *e.g.,* rheumatoid arthritis, multiple sclerosis and psoriasis. Furthermore, since transcription of hepatitis B core and X proteins as well as the long terminal repeat of human HIV-1 are controlled by HNF-4$_\alpha$, HNF-4$_\alpha$ could be involved in modulating the course of infection initiated by these viral agents.

Since overexpression of HNF-4$_\alpha$-induced genes may result in dyslipoproteinemia, IGT/NIDDM, hypertension, blood coagulability and fibrinolytic defects, atherogenesis, cancer, inflammatory, immunodeficiency and other diseases, inhibition of HNF-4$_\alpha$ transcriptional activity may be expected to result in amelioration of HNF-4$_\alpha$-induced pathologies. However, no ligand has yet been identified for HNF-4$_\alpha$ which could serve as basis for designing inhibitors of HNF-4$_\alpha$ transcriptional activity. This invention is concerned with low molecular weight ligands of HNF-4$_\alpha$ designed to act as inhibitors of HNF-4$_\alpha$-induced transcription and therefore as potential drugs in the treatment of pathologies induced by or involving HNF-4$_\alpha$-controlled genes.

The Invention

a. Long chain acyl-CoAs are ligands for HNF-4

Fatty acyl-CoAs of various chain length and degree of saturation were found to specifically bind to HNF-4. Binding was exemplified with either the ligand binding domain of HNF-4 fused to glutathione-s-transferase (GST-HNF-4(LBD)) or the full length HNF-4 protein tagged by 6 histidines (His-HNF-4). Palmitoyl(16:0)-CoA binding to the ligand binding domain or full length HNF-4 proteins was saturable having a Kd of 2.6 M and approaching at saturation a ratio of 1 mole of fatty acyl-CoA/mole of HNF-4 (Fig. 1A). Binding was specific for the acyl-CoA whereas the free fatty acid or free CoA were inactive. The binding of fatty acyl-CoAs of variable chain length and degree of saturation was verified by competing with radiolabelled palmitoyl(16:0)-CoA binding to recombinant GST-HNF-4(LBD) or His-HNF-4 (Fig. 1B). Binding was not observed with saturated fatty acyl-CoAs shorter than C12 in chain length. However, the binding affinity of long chain fatty acyl-CoAs for HNF-4 was not substantially affected by chain length or degree of saturation of respective ligands, being in the range of 0.5 3.0 M. Specificity of binding of long chain fatty acyl-CoAs to HNF-4 was further verified by analyzing the putative binding of palmitoyl(16:0)-CoA to recombinant histidine-tagged peroxisome proliferators activated receptor (His-PPAR). In contrast to HNF-4, long chain fatty acyl-CoAs were not bound by PPAR. These results indicate that natural long chain fatty acyl-CoAs may bind to the ligand binding domain of HNF-4 and serve as specific ligands of this protein.

Binding of acyl-COAs to HNF-4 is not limited to natural fatty acyl-CoAs as exemplified above. Thus, binding may be observed with xenobiotic acyl-CoAs (RCOSCoA) where R is a radical consisting of a saturated or unsaturated alkyl

chain of 10 24 carbon atoms, one or more of which may be replaced by heteroatom, where one or more of said

carbon or heteroatom chain members optionally forming part of a ring, and where said chain being optionally substituted.

b. **Modulation of HNF-4$_{\alpha}$ activity by long chain acyl-CoAs**

HNF-4$_{\alpha}$ activity as a function of binding of long chain acyl-CoAs was evaluated by studying the binding of HNF-4$_{\alpha}$ to its cognate C3P element of the apo CIII promoter sequence (87/ 66)[6] in the presence or absence of added acyl-CoAs of variable chain length, degree of saturation, and degree of substitution. Binding was verified by using a gel mobility shift assay. As shown in Fig. 2A, C3P binding to HNF-4$_{\alpha}$ increased with increasing His-HNF-4$_{\alpha}$ concentrations and was activated by natural saturated fatty acyl-CoAs of C12 C16 in chain length. Activation was concentration dependent and maximal in the presence of myristoyl(14:0)-CoA added within a concentration range required for its binding to HNF-4$_{\alpha}$. Monounsaturated (oleoyl(18:1)-CoA), (w-6)polyunsaturated (linoleoyl(18:2)-CoA) or (w-3)polyunsaturated (eicosapentaenoyl(20:5)-CoA, docosahexaenoyl(22:6)-CoA) fatty acyl-CoAs were only partially active as compared with myristoyl(14:0)-CoA (Fig. 2B). Furthermore, some fatty acyl-CoAs as well as xenobiotic acyl-CoAs were found to serve as true antagonists of HNF-4$_{\alpha}$, namely to inhibit its intrinsic binding to its cognate enhancer. Thus, incubating HNF-4$_{\alpha}$ in the presence of either stearoyl(18:0)-CoA or $_{\alpha}$-linolenoyl(18:3)-CoA resulted in potent inhibition of its binding to C3P oligo-nucleotide (Fig. 2C). Similarly, incubating HNF-4$_{\alpha}$ in the presence of a variety of xenobiotic acyl-CoAs resulted in inhibition of its binding to its cognate C3P oligonucleotide. Hence, natural or xenobiotic acyl-CoAs which bind to HNF-4$_{\alpha}$ may serve as agonists, partial agonists or antagonists of its transcriptional activity as a function of chain length, degree of saturation or degree of substitution.

c. M dulation of HNF-4$_\alpha$-induced transcription by HNF-4$_\alpha$ agonists and antagonists

The effect of agonistic and antagonistic HNF-4$_\alpha$-ligands was further evaluated by analyzing the *in vitro* transcription rate, catalyzed by added HeLa nuclear extract and induced by recombinant HNF-4$_\alpha$, of a test template consisting of a 377 bp G-less cassette promoted by sequences of the HSV thymidine kinase and chicken ovalbumin promoters and enhanced by three C3P copies of the apo CIII gene promoter. Transcriptional activation by HNF-4$_\alpha$ was evaluated in the presence and in the absence of added representative long chain fatty acyl-CoAs. Transcription of a template consisting of a 200 bp G-less cassette driven by the adenovirus major late (AdML) promoter and lacking an HNF-4$_\alpha$ enhancer was used as an internal control template. As shown in Fig. 3, *in vitro* transcription of the test template increased as a function of HNF-4$_\alpha$, approaching saturation at HNF-4$_\alpha$ concentrations of 200 ng. HNF-4$_\alpha$ induced transcription was activated by added palmitoyl(16:0)-CoA and inhibited by added stearoyl(18:0)-CoA in line with the effect exerted by HNF-4$_\alpha$ agonists and antagonists in gel mobility shift assays. Hence, acyl-CoAs which bind to HNF-4$_\alpha$ may directly modulate its transcriptional activity in a cell free system.

The intracellular effect of HNF-4$_\alpha$ ligands on HNF-4$_\alpha$ mediated transcription was evaluated in COS-7 cells cotransfected with an expression vector for HNF-4$_\alpha$ and with a CAT reporter plasmid driven by a thymidine kinase promoter and enhanced by one to three C3P copies of the apo CIII gene promoter. Transfected cells were incubated in the presence of free fatty acids and xenobiotic amphipathic carboxylates representing agonistic or antagonistic HNF-4$_\alpha$ ***proligands***. As shown in Fig. 4a, expression of the C3P-enhanced reporter plasmid was 7 fold activated by HNF-4$_\alpha$ in the absence of added fatty acids to the culture medium. Transcriptional activation by transfected HNF-4$_\alpha$ could reflect the intrinsic transcriptional activity of the unliganded HNF-4$_\alpha$ dimer or could result from binding to HNF-4$_\alpha$ of an endogenous activatory acyl-CoA. Adding palmitic(16:0) acid to

the culture medium resulted in dose dependent activation of HNF-4_ dependent transcription whereas stearic(18:0) or _-linolenic(18:3) acids were suppressive in line with the agonistic or antagonistic activities of the respective fatty acyl-CoAs in gel mobility shift assays (Fig. 2A,C) as well as in cell free transcription assays (Fig. 3). In contrast to the partial agonistic activities of linoleoyl(18:2)-CoA or eicosapentaenoyl(20:5)-CoA in DNA binding studies (Fig. 2B), adding linoleic(18:2) or eicosapentaenoic(20:5) acids to the culture medium resulted in inhibition of HNF-4_ induced transcription which may be ascribed to substituting of an endogenous HNF-4_ agonist by an exogenously added partial agonist. Inhibition of cellular transcription by eicosapentaenoic(20:5) acid was significantly more pronounced as compared with that by linoleic(18:2) acid, and was comparable with that induced by true HNF-4_ antagonists. Inhibition of HNF-4_ transcriptional activity in transfection assays may be similarly observed in the presence of added xenobiotic amphipathic carboxylates (RCOOH) to the culture medium (Fig. 4b) where R is a radical consisting of a saturated or unsaturated alkyl chain of 10 24 carbon atoms, one or more of which may be replaced by heteroatom, where one or more of said carbon or heteroatom chain members optionally forming part of a ring, and where said chain being optionally substituted by hydrocarbyl radical, heterocyclyl radical, lower alkoxy, hydroxyl-substituted lower alkyl, hydroxyl, carboxyl, halogen, phenyl, substituted phenyl, C C cycloalkyl or substituted C C cycloalkyl. Hence, intracellular HNF-4_-mediated expression may be modulated by natural long chain fatty acids as well as by xenobiotic amphipathic carboxylates capable of being endogenously converted to their respective CoA thioesters (RCOSCoA). The overall effect exerted may reflect the prevailing composition of nuclear acyl-CoAs and the agonistic/antagonistic effect exerted by respective HNF-4_ ligands.

Highly effective inhibitory comp unds are th following wher R is substitut d by -carboxyl:

1,16 Hexadecanedioic acid
1,18 Octadecanedioic acid
2,2,15,15-tetramethyl-hexadecane-1,16-dioic acid
2,2,17,17-tetramethyl-octadecane-1,18-dioic acid
3,3,14,14-tetramethyl-hexadecane-1,16-dioic acid
3,3,16,16-tetramethyl-octadecane-1,18-dioic acid
4,4,13,13-tetramethyl-hexadecane-1,16-dioic acid
4,4,15,15-tetramethyl-octadecane-1,16-dioic acid

Another group of highly effective compounds is that in which R is substituted by -hydroxy boron:

16-B (OH) -hexadecanoic acid
18-B (OH) -octadecanoic acid
16-B (OH)2-2,2-dimethyl-hexadecanoic acid
18-B (OH)2-2,2-dimethyl-octadecanoic acid
16-B (OH)2-3,3-dimethyl-hexadecanoic acid
18-B (OH)2-3,3-dimethyl-octadecanoic acid
16-B (OH)2-4,4-dimethyl-hexadecanoic acid
18-B (OH)2-4,4-dimethyl-octadecanoic acid
2
2

Yet another group of highly effective compounds is that of compounds wherein R is substituted by -hydroxyl:

16-hydroxy-hexadecanoic acid
18-hydroxy-octadecanoic acid
16-hydroxy-2,2-dimethyl-hexadecanoic acid
18-hydroxy-2,2-dimethyl-octadecanoic acid
16-hydroxy-3,3-dimethyl-hexadecanoic acid
18-hydroxy-3,3-dimethyl-octadecanoic acid
16-hydroxy-4,4-dimethyl-hexadecanoic acid
18-hydroxy-4,4-dimethyl-octadecanoic acid

d. Physiological relevance

Inhibition of HNF-4$_\alpha$ transcriptional activity by natural or xenobiotic amphipathic carboxylate capable of being endogenously converted to their respective CoA thioesters may offer a therapeutic mode for treating diseases initiated and/or promoted by overexpression of HNF-4$_\alpha$ controlled genes. The performance of a concerned amphipathic carboxylate as inhibitor of HNF-4$_\alpha$ transcriptional activity will depend in the first place on the ***intrinsic*** capacity of its respective CoA thioester to act as HNF-4$_\alpha$ antagonist. Presently it is impossible to predict which amphipathic carboxylates capable of being endogenously converted to their respective CoA thioesters may prove as true antagonists of HNF-4$_\alpha$. Thus, myristoyl(14:0)-CoA or palmitoyl(16:0)-CoA proved as activators of HNF-4$_\alpha$ transcriptional activity while the next homologue in the series, namely stearoyl(18:0)-CoA proved a true antagonist. It should be pointed out however that partial agonists may induce an apparent inhibition of HNF-4$_\alpha$ activity if substituting for endogenous HNF-4$_\alpha$ potent agonists or if competing with more productive agonists for binding to HNF-4$_\alpha$. Thus, partial agonists like eicosapentaenoyl(20:5)-CoA or docosahexaenoyl(22:6)-CoA do act *in vivo* as inhibitors of HNF-4$_\alpha$ transcriptional activity (Fig. 4a) due presumably to substituting for endogenous HNF-4$_\alpha$ agonists like myristoyl(14:0)-CoA or palmitoyl(16:0)-CoA.

The overall *in vivo* performance of an amphipathic carboxylate as an inhibitor of HNF-4$_\alpha$ transcriptional activity may not only reflect the intrinsic capacity of its respective CoA thioester to act as HNF-4$_\alpha$ antagonist, but will further depend on the specific cell type and the prevailing composition of nuclear fatty acyl-CoAs. This composition may be affected by the dietary/ pharmacological availability/profile of respective acids, the availability of each for CoA-thioesterification as well as the availability of respective acyl CoAs for hydrolysis by acyl-CoA hydrolases, esterification into lipids, oxidation into products, elongation, desaturation or binding to other acyl-CoA binding proteins. Furthermore, endogenous acyl-CoAs produced by CoA-thioesterification of amphipathic carboxylates other than fatty acids (*e.g.*,

retinoic acid, prostaglandins, leukotrienes, others) could bind to HNF-4$_\alpha$ and modulate its activity as agonists or antagonists. The resultant effect may further depend on additional nuclear factors which may influence the oligomeric-dimeric equilibrium of HNF-4$_\alpha$, the binding affinity of HNF-4$_\alpha$ to its cognate enhancer or the interaction between HNF-4$_\alpha$ and proteins of the transcriptional initiation complex. In particular, since HNF-4$_\alpha$ and the peroxisomal activators activated receptor (PPAR) share similar DR-1 consensus sequences, and as PPAR may be activated by long chain free fatty acids rather than their respective CoA thioesters, the effect exerted by a certain acyl-CoA and mediated by HNF-4$_\alpha$ could be either similar to or antagonized by PPAR activated by the respective free acid.

In spite of the above unknowns, the agonistic/antagonistic profile of acyl-CoA ligands of HNF-4$_\alpha$ as exemplified here may help in realizing the molecular basis of effects exerted by dietary fatty acids *in vivo* and concerned with some of the genes regulated by HNF-4$_\alpha$. Long chain fatty acyl constituents of dietary fat comprise 30 40% of the caloric intake of Western diets. In addition to their substrate role, being mostly oxidized to yield energy or esterified into triglycerides and phospholipids to yield adipose fat and cell membranes, respectively, some dietary fatty acids have long been realized as neutriceutical modulators of the onset and progression of cancer[7], atherogenesis[8], dyslipoproteinemia[9], insulin resistance[10,11], hypertension[12], blood coagulability and fibrinolytic defects[13], inflammatory, immunodeficiency and other diseases. These unexplained effects may now be realized to be accounted for by the effect exerted by the respective acyl-CoAs on HNF-4$_\alpha$ transcriptional activity resulting in modulating the expression of genes involved in the onset and progression of the above pathologies. The specific effects exerted by dietary long chain fatty acids on blood lipids and blood coagulation are worth noting in light of the well established effect exerted by HNF-4$_\alpha$ on genes coding for proteins involved in lipoproteins metabolism (apolipoproteins AI, AII, B, CIII, microsomal triglyceride transfer protein) and blood coagulation (factors IV, IX, X). Indeed, the well established increase in plasma VLDL-, LDL- and HDL-cholesterol

induced by dietary saturated fatty acids of C12 C16 in general and by myristic acid in particular is in line with HNF-4$_\alpha$ activation induced by the respective saturated acyl-CoAs and the lack of effect exerted by fatty acyl-CoAs shorter than C12. The surprisingly lowering of blood lipids by the saturated stearic(18:0) acid may be similarly accounted for by the antagonistic effect exerted by stearoyl(18:0)-CoA on HNF-4$_\alpha$ activity. Similarly, the lipid lowering effect of mono and polyunsaturated fatty acids, ascribed to substituting for saturated dietary fatty acids[9], is in line with the partial agonistic activity of poly or monounsaturated as compared with saturated fatty acyl-CoAs, being further complemented by the direct inhibition of HNF-4$_\alpha$ by linolenoyl(18:3)-CoA. Also, the increase in blood coagulability induced by saturated C12 C16 dietary fatty acids and correlated with a respective increase in factor VII, the decrease in coagulability induced by polyunsaturated dietary fatty acids as well as the surprising decrease in factor VII content and blood coagulability specifically induced by dietary stearic(18:0) acid may be similarly ascribed to the effect exerted by the respective fatty acyl-CoAs on HNF-4$_\alpha$ activity resulting in modulating the expression of HNF-4$_\alpha$-controlled genes encoding vitamin K-dependent coagulability factors.

Furthermore, modulation of transcription of HNF-4$_\alpha$-controlled genes by xenobiotic amphipathic carboxylates which may endogenously be esterified to their respective CoA thioesters and act as HNF-4$_\alpha$ agonists or antagonists may offer a pharmacological therapeutic mode for diseases initiated or promoted by overexpression of HNF-4$_\alpha$-controlled genes. The examples offered by xenobiotic substituted amphipathic dicarboxylates are worth noting in light of the cumulative information concerned with their pharmacological performance in changing the course of dyslipoproteinemia, obesity, insulin resistance and atherosclerosis in animal models[14 17], namely, of diseases concerned with overexpression of some HNF-4$_\alpha$-controlled genes. The therapeutic efficacy of these drugs may be accounted for by inhibition of HNF-4$_\alpha$ transcriptional activity as exemplified here.

Methods

HNF-4 Recombinant Proteins

Rat HNF-4$_1$ cDNA(pLEN4S)[1] was subcloned into the glutathione-S-transferase (GST) encoding pGEX-2T plasmid (Pharmacia) and the resultant plasmid was cleaved with smaI and AccI and religated to yield the GST-HNF-4 (LBD) fusion plasmid. The fusion plasmid was expressed in *E.coli* BL21(DE3) strain by induction with 0.2 mM IPTG for 60 min and the product was purified by affinity chromatography using glutathione-agarose beads (Sigma) to yield the GST-HNF-4 (LBD) fusion protein consisting of amino acids 96-455 of wild type HNF-4 fused to GST. The full length HNF-4$_1$ cDNA cloned into 6His-pET11d vector was expressed in *E.coli* BL21(DE3)plysS.

Ligand Binding Assays

Recombinant GST-HNF-4 (LBD) (100 pmol) or His-HNF-4$_{100}$ pmol) were incubated for 60 min at 22°C with [^{3}H]palmitoyl(16:0)-CoA (American Radiolabeled Chemicals) in 100 l of 10 mM phosphate buffer (pH 7.4). Competitor ligands or solvent carrier were added as indicated. Free and HNF-4 bound ^{3}[H]palmitoyl(16:0)-CoA were separated by Dowex-coated charcoal and bound ligand was quantified by liquid scintillation counting. Nonspecific binding of [^{3}H]palmitoyl(16:0)-CoA was determined by its binding to the GST moiety or to carbonic anhydrase as nonrelevant protein.

Gel Mobility Shift Assays

His-HNF-4 and acyl-CoA (as indicated) were preincubated for 30 min at 22°C in 11 mM Hepes (pH 7.9) containing 50 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl , 10% glycerol, 1 g of poly(dI-dC) in a final volume of 20 l. ^{32}P-labeled oligonucleotide (0.1 ng) consisting of the human C3P apo CIII promoter sequence (87/ 66)[6] was then added, and incubation was continued for an additional 15 min. Protein-DNA complexes were resolved by 5% non-denaturing polyacrylamide gel in 0.6 TBE and quantitated by PhosphorImager analysis.

***In Vitro* Transcription Assays**

Reaction mixture contained 20 mM Hepes-KOH (pH 7.9), 5 mM MgCl , 60 mM KCl, 8% glycerol, 2 mM DTT, 1 mM 3 -0-methyl-GTP, 10 units of T1 RNase, 20 units of RNasin, 0.5 g sonicated salmon sperm DNA and His-HNF-4 and test ligand as indicated. The mixture was preincubated for 30 min at 22°C followed by adding 10 ng of pAdML200 control template consisting of the adenovirus major late promoter (400/+10) linked to a 200 bp G-less cassette and 200 ng of the test template consisting of three C3P copies of the apo CIII promoter sequence (87/ 66) upstream to a synthetic ovalbumin TATA box promoter in front of a 377 bp-G-less cassette. The mixture was further preincubated for 10 min at 22°C followed by adding 40 g of HeLa nuclear extract with additional preincubation for 30 min at 30°C. 0.5 mM ATP, 0.5 mM CTP, 25 M UTP, and 10 Ci of [-^{32}P]UTP (s.a. 800 Ci/mol, Amersham) were then added and the complete reaction mixture was incubated for 45 min at 30°C in a final volume of 25 l. The reaction was terminated by adding 175 l of stop mix (0.1 M sodium acetate (pH 5.2), 10 mM EDTA, 0.1% SDS, 200 g/ml tRNA) followed by phenol extraction and ethanol precipitation. RNA was resuspended in sample buffer containing 80% formamide and 10 mM Tris-HCl (pH 7.4) and separated on 5% polyacrylamide gel containing 7 M urea in TBE. Correctly initiated transcripts were quantitated by PhosphorImager analysis. The test DNA template was constructed by inserting into pC AT19 plasmid a PCR-amplified oligonucleotide prepared by using the (C3P) -TK-CAT plasmid as template and consisting of three copies of the C3P element of the Apo CIII promoter sequence (87/ 66) having an ECoRI and SSTI sites at the 5 and 3 ends, respectively. The resultant plasmid was cleaved with sphI and sacI and ligated to a synthetic oligo-nucleotide (**5 -CGAGGTCCAC-TTCGCTATATATTCCCCGAGCT-3**) containing sequences of the HSV thymidine kinase promoter (41/ 29) and of the chicken ovalbumin promoter (33/ 21).

Transfection Assays

COS-7 cells cotransfected for 6 h with the (C3P) -TK-CAT reporter plasmid (5 g) and with either the pSG5-HNF-4 expression plasmid (0.025 g) or the pSG5 plasmid (0.025 g) added by calcium phosphate precipitation were cultured in serum free medium with fatty acids (complexed with albumin in a molar ratio of 6:1) added as indicated. -Galactosidase expression vector pRSGAL (1 g) added to each precipitate served as an internal control for transfection. The (C3P) -TK-CAT construct was prepared by inserting a synthetic oligonucleotide encompassing the (87/ 66) Apo CIII promoter sequence (**5 -GCAGGTGACCTTTGCCCAGCGCC-3**) flanked by HindIII restriction site into pBLCAT2[47] upstream of the 105 bp thymidine kinase promoter. The construct containing three copies of the synthetic oligonucleotide in the direct orientation was selected and confirmed by sequencing.

Fatty Acyl-CoAs

Fatty acyl-CoAs were prepared by reacting the free acid dissolved in dry acetonitrile with 1,1 -carbonyldiimidazole. The reaction mixture was evaporated to dryness and the respective acyl-imidazole conjugate was reacted with CoA dissolved in 1:1 THF:H O. Reaction was followed by TLC using silica 60H plates (Merck) (butanol: acetic acid:H O 5:2:3). The acyl-CoA derivative was precipitated with 0.1 M HCl and the precipitate was washed three times with 0.1 M HCl, three times with peroxide free ether and three times with acetone. The acyl-CoA was spectrophotometrically determined by its 260/232 nm ratio.

Legends to Figures

FIG. 1 Long chain acyl-CoAs are ligands for HNF-4_. The GST-HNF-4_(LBD) fusion protein ( ) consists of HNF-4_ (LBD) fused to glutathione-S transferase. The His-HNF-4_ ( ) consists of the full length HNF-4_ tagged by 6 histidines.

a. Saturation binding curve for palmitoyl(16:0)-CoA. The respective recombinant proteins are incubated to equilibrium with [^{3}H]palmitoyl(16:0)-CoA (0.05 Ci) and with increasing nonlabeled palmitoyl(16:0)-CoA as indicated. A dissociation constant (Kd) of 2.6 M and maximal binding of 1 mol palmitoyl(16:0)-CoA/mol HNF-4_ are determined by Scatchard analysis (inset).

b. Competition by myristoyl(14:0)-CoA. The respective recombinant proteins are incubated with 8 nM of [^{3}H]palmitoyl(16:0)-CoA (60 Ci/mmol) and with increasing nonlabeled myristoy(14:0)-CoA as indicated. Percent bound refers to radiolabeled [^{3}H]palmitoyl(16:0)-CoA in the bound fraction. 100% binding amounts to 0.3 pmol of [^{3}H]palmitoyl(16:0)-CoA. Percent found refers to radiolabeled [^{3}H]palmitoyl(16:0)-CoA in the bound fraction. 100% binding amounts to 0.3 pmol of [^{3}H]palmitoyl(16:0)-CoA. EC (50% specific competition) amounts to 1.4 M (range 1.2 1.5 M) of myristoyl(14:0)-CoA. EC for other fatty acyl-CoAs are as follows:

Dodecanoyl(12:0)-CoA	2.3 M (range 2.1 2.4 M);
Palmitoyl(16:0)-CoA	2.6 M (range 1.3 3.4 M);
Stearoyl(18:0)-CoA	2.7 M (range 2.1 3.3 M);
Oleoyl(18:1)-CoA	1.4 M (range 1.0 1.8 M);
Linoleoyl(18:2)-CoA	1.9 M (range 1.5 2.3 M);
Linolenoyl(18:3)-CoA	2.9 M (range 2.9 3.8 M);
Eicosapentaenoyl(20:5)-CoA	0.6 M (range 0.5 0.7 M);
Docosahexaenoyl(22:6)-CoA	1.6 M (range 0.6 2.7 M).

EC for other xenobiotic acyl-CoA examples are esentially similar to the above.

FIG. 2 Fatty acyl-CoA ligands of HNF-4$_{\alpha}$ modulate its binding to its cognate DNA enhancer.

a. Activation of HNF-4$_{\alpha}$ binding to its C3P cognate enhancer by added long chain saturated fatty acyl-CoAs. The gel section containing radiolabeled C3P bound to His-HNF-4$_{\alpha}$ dimer is shown. Each ligand is evaluated using either 14 ng (odd-number lanes) or 20 ng (even number lanes) of His-HNF-4$_{\alpha}$. Lanes 1 and 2 show binding of radiolabeled C3P to His-HNF-4$_{\alpha}$ in the absence of added fatty acyl-CoA. Lanes 3 to 8 show activation of binding by 10 M each of dodecanoyl(12:0)-CoA, myristoyl(14:0)-CoA or palmitoyl(16:0)-CoA. Activation of binding by myristoyl(14:0)-CoA is dose dependent (lanes 9 16). No activation of binding is observed with 10 M of free myristic(14:0) acid (lane 17).

b. Activation of HNF-4$_{\alpha}$ binding to its C3P cognate enhancer by mono- and polyunsaturated fatty acyl-CoAs. Each ligand is evaluated using either 20 ng (odd number lanes) or 29 ng (even number lanes) of His-HNF-4$_{\alpha}$. Lanes 1 to 8 show activation of binding by 10 M each of oleoyl(18:1)-CoA and linoleoyl(18:2)-CoA as compared with myristoyl(14:0)-CoA. Lanes 9 to 16 show activation of binding by 10 M each of eicosapentaenoyl(20:5)-CoA and docosahexa-enoyl(22:6)-CoA as compared with myristoyl(14:0)-CoA.

c. Inhibition of HNF-4$_{30)\ \alpha}$ ng) binding to its C3P cognate enhancer by 10 M each of stearoyl(18:0)-CoA or linolenoyl(18:3)-CoA as compared with HNF-4$_{\alpha}$ activation by 10 M of myristoyl(14:0)-CoA.

FIG. 3 Modulation of HNF-4_ transcriptional activity by long chain fatty acyl-CoAs *in vitro*.

a. Representative experiments showing *in vitro* transcription of the test template in the presence of increasing concentrations of His-HNF-4_ and in the absence (lanes 1 3, 7 9) or presence of 10 M of added palmitoyl(16:0)-CoA (lanes 4 6) or stearoyl(18:0)-CoA (lanes 10,11) as indicated. Correctly initiated transcripts of the test and control templates are denoted by (_) and (-_), respectively.

b. HNF-4_-induced *in vitro* transcription in the absence ( ) or presence of 10 M each of added palmitoyl(16:0)-CoA ( ) or stearoyl(18:0)-CoA ( ). Fold transcription indicates the ratio of specific transcript produced by the test template over transcript from the control template normalized to the ratio observed without HNF-4_. The figure summarizes 5 independent experiments for each acyl-CoA. *-Significant as compared with the respective value in the absence of added ligand.

FIG. 4 Modulation of HNF-4_ activity by long chain fatty acids and xenobiotic amphipathic carboxylates in transient transfection assays. Fold induction of CAT activity by transfected HNF-4_ is determined by evaluating CAT activity in the presence of pSG5-HNF-4_ as compared with pSG5 plasmid and as function of respective fatty acids added to the culture medium as indicated. The figure summarizes 3 4 independent experiments for each fatty acid. Mean ± S.E.

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CLAIMS

1. A pharmaceutical composition for the treatment or prophylaxis of increased levels of cholesterol in the blood, of increased triglycerides, of dyslipo-proteinemia or of low levels of HDL-cholesterol, containing an effective quantity of a prodrug of a pharmacologically active compound of the formula RCOSCoA, into which the said prodrug is converted, where CoA designates coenzyme A, where the prodrug is an amphipathic carboxylate of the formula R-COOH, or a salt or an ester or amide of such compound, where R designates a saturated or unsaturated alkyl chain of 10-24 carbon atoms, one or more of which may be replaced by heteroatoms, where one or more of said carbon or heteroatom chain members optionally forms part of a ring, and where said chain is optionally substituted by a hydrocarbyl radical, heterocyclyl radical, lower alkoxy, hydroxyl-substituted lower alkyl, hydroxyl, carboxyl halogen, phenyl or (hydroxy- lower alkyl, lower alkoxy-, lower alkenyl- or lower alkinyl)-substituted phenyl, C_3-C_7 cycloalkyl or (hydroxy-, lower alkyl-, lower alkoxy-, lower alkenyl- or lower alkinyl)-substituted C_3-C_7 cycloalkyl with the proviso that compounds of the formula RCOOH which are known as active for an indications similar to that set out above are not claimed.

2. Compositions according to claim 1, where in the compound R is substituted by ω-carboxyl, ω-hydroxyl boron or by ω-hydroxyl.

3. A composition according to claim 1, where R is a long chain acyl-CoA compound.

4. A composition according to claim 2, where the fatty acyl-CoA compound is chosen from:

   Stearoyl(18:0)-CoA
   Oleoyl(18:1)-CoA
   Linoleoyl(18:2)-CoA
   Linolenoyl(18:3)-CoA
   Eicosapentaenoyl(20:5)-CoA
   Docosahexaenoyl(22:6)-CoA

5. A composition according to claim 1, where in the effective compound is chosen from the following compounds, where R is substituted by ω-carboxyl:

1,16 Hexadecanedioic acid
1,18 Octadecanedioic acid
2,2,15,15-tetramethyl-hexadecane-1,16-dioic acid
2,2,17,17-tetramethyl-octadecane-1,18-dioic acid
3,3,14,14-tetramethyl-hexadecane-1,16-dioic acid
3,3,16,16--tetramethyl-octadecane-1,18-dioic acid
4,4,13,13-tetramethyl-hexadecane-1,16-dioic acid
4,4,15,15-tetramethyl-octadecane-1,16-dioic acid

6. A composition according to claim 1, where R is substituted by ω-hydroxy boron:

16-B (OH)2-hexadecanoic acid
18-B (OH)2-octadecanoic acid
16-B (OH)2-2,2-dimethyl-hexadecanoic acid
18-B (OH)2-2,2-dimethyl-octadecanoic acid
16-B (OH)2-3,3-dimethyl-hexadecanoic acid
18-B (OH)2-3,3-dimethyl-octadecanoic acid
16-B (OH)2-4,4-dimethyl-hexadecanoic acid
18-B (OH)2-4,4-dimethyl-octadecanoic acid

7. A composition according to claim 1, where R is substituted by ω-hydroxy:

16-hydroxy-hexadecanoic acid
18-hydroxy-octadecanoic acid
16-hydroxy-2,2-dimethyl-hexadecanoic acid
18-hydroxy-2,2-dimethyl-octadecanoic acid
16-hydroxy-3,3-dimethyl-hexadecanoic acid
18-hydroxy-3,3-dimethyl-octadecanoic acid
16-hydroxy-4,4-dimethyl-hexadecanoic acid
18-hydroxy-4,4-dimethyl-octadecanoic acid

8. A composition according to any of claims 1 to 7, for the treatment of insulin resistance, impaired glucose tolerance and NIDDM.

9. A composition according to any of claims 1 to 7, for the treatment of essential hypertension.

10. A composition according to any of claims 1 to 8, for reducing blood coagulability or increasing blood fibrinolysis.

11. A composition according to any of claims 1 to 8, for the treatment of the metabolic syndrome (Syndrome-X).

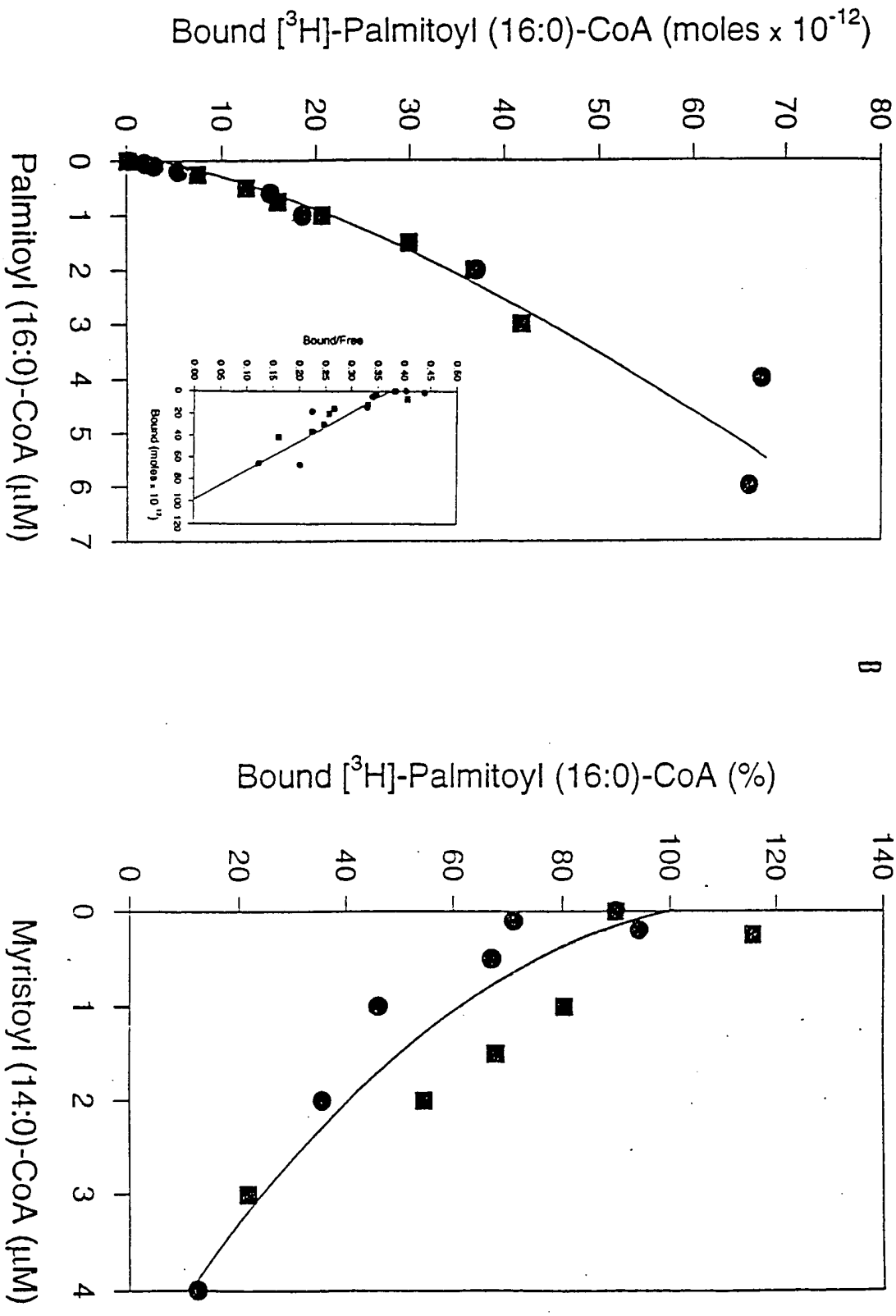
12. A composition according to any of claims 1 to 8, for the treatment of coronary or peripheral atherosclerosis.

13. A composition according to any of claims 1 to 8, for the treatment of rheumatoid arthritis, multiple sclerosis of psoriasis.

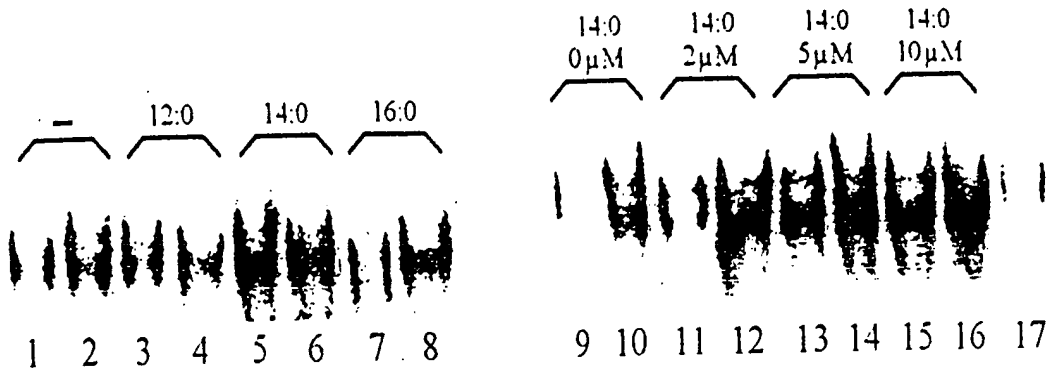
14. A composition according to any of claims 1 to 8, for the treatment of breast cancer, colon cancer, or prostate cancer.

15. A pharmaceutical composition according to any of claims 1 to 14, for topical use.

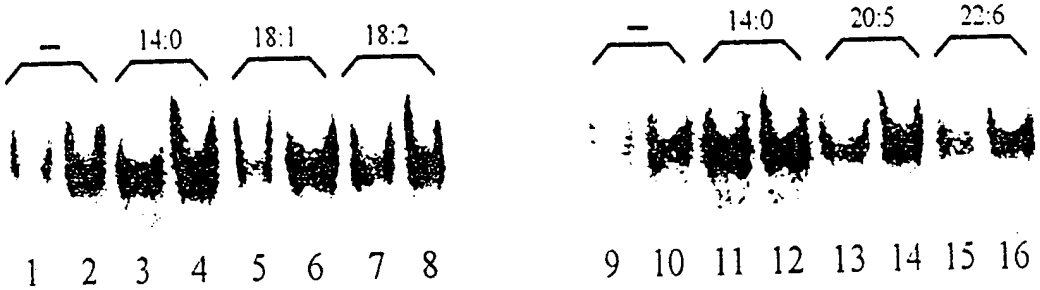
FIG. 1


A
Bound [3H]-Palmitoyl (16:0)-CoA (moles x 10-12)
0
10
20
30
40
50
60
70
80
Palmitoyl (16:0)-CoA (µM)
0
1
2
3
4
5
6
7
Bound/Free
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
Bound (moles x 10-12)
0
20
40
60
80
100
120
B
Bound [3H]-Palmitoyl (16:0)-CoA (%)
0
20
40
60
80
100
120
140
Myristoyl (14:0)-CoA (µM)
0
1
2
3
4

A


–
12:0
14:0
16:0
14:0 0μM
14:0 2μM
14:0 5μM
14:0 10μM
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16 17

B


–
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18:1
18:2
–
14:0
20:5
22:6
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16

C

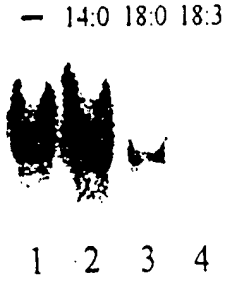
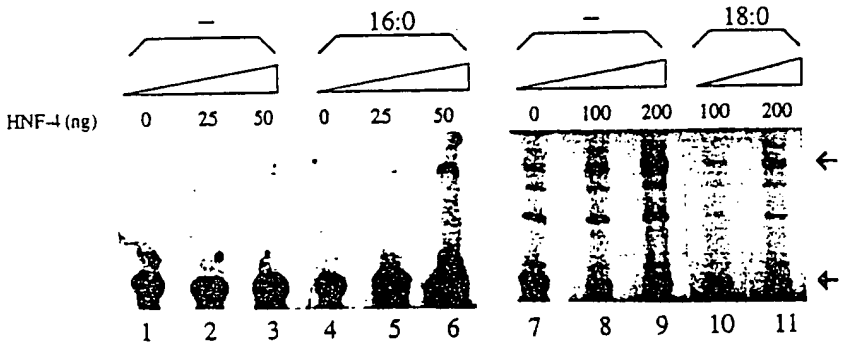
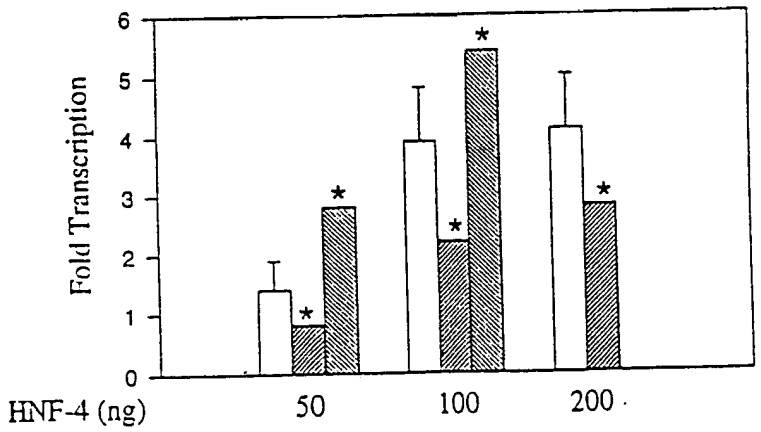

–
14:0
18:0
18:3
1 2 3 4

FIG. 3

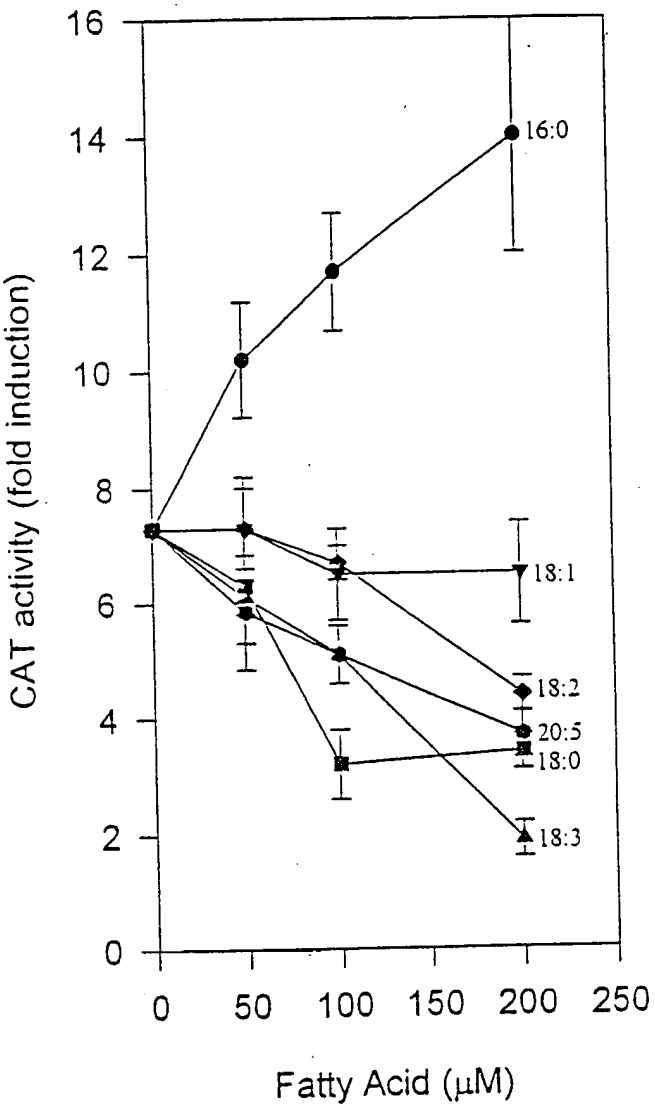
A


–
16:0
–
18:0
HNF-4 (ng)
0 25 50 0 25 50 0 100 200 100 200
1 2 3 4 5 6 7 8 9 10 11

B


Fold Transcription
0 1 2 3 4 5 6
HNF-4 (ng)
50 100 200

A


16
14
12
10
8
6
4
2
0
CAT activity (fold induction)
16:0
18:1
18:2
20:5
18:0
18:3
0
50
100
150
200
250
Fatty Acid (μM)